

RESEARCH ARTICLE

## A Recessive Arabidopsis Mutant That Grows Photoautotrophically under Salt Stress Shows Enhanced Active Oxygen Detoxification

Kazuo Tsugane,<sup>a</sup> Kyoko Kobayashi,<sup>a</sup> Yasuo Niwa,<sup>a</sup> Yasushi Ohba,<sup>b</sup> Keishiro Wada,<sup>b</sup> and Hirokazu Kobayashi<sup>a,1</sup>

<sup>a</sup>Laboratory of Plant Cell Technology, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

<sup>b</sup>Department of Biology, Faculty of Science, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan

Mutagenized Arabidopsis seedlings (ecotype Columbia) were screened for the ability to grow photoautotrophically on solid medium containing 200 mM NaCl. A novel mutant line, designated *pst1* (for *photoautotrophic salt tolerance1*), was obtained. There were no significant differences between *pst1* and wild-type plants with regard to their ability to induce proline as an osmoregulatory solute. In addition, the content of monovalent cations in *pst1* plants grown with or without salt stress was equal to that in the wild type. We observed that light, even at moderate intensities, increased the effects of salt stress on wild-type plants. The *pst1* seedlings were nearly 10 times more tolerant to methyl viologen than were wild-type seedlings. We also found that the activities of the active oxygen scavengers superoxide dismutase and ascorbate peroxidase were enhanced significantly in *pst1* plants. The *pst1* plants also were tolerant to other stresses, such as high light intensity and toxic monovalent cations. The recessive nature of the *pst1* mutation indicates that the potential for salt-stress tolerance is blocked in wild-type Arabidopsis.

### INTRODUCTION

Arid areas are expanding gradually but steadily on earth. It is known that salt accumulation caused by unsuitable irrigation practices decreases crop productivity (Epstein et al., 1980). Under these conditions, high concentrations of NaCl arrest plant development and lead to plant death. Several interacting events are triggered in plants by salt stress, including the inhibition of enzyme activities in metabolic pathways, decreased uptake of nutrients in roots, decreased carbon-use efficiency, and the denaturation of protein and membrane structures. However, the complete response of plants to salt stress has not been explained systematically (Benzel and Reuveni, 1994).

Several mechanisms of defense and adaptation to water deficiency resulting from salt stress have been well studied. Adaptation to water deficiency can involve the accumulation of osmolytes, such as proline (Igarashi et al., 1997), glycinebetaine, and sugar alcohols (Bohnert et al., 1995), or late-embryo-abundant (Lea) proteins (Dure, 1993). With this knowledge, researchers have attempted to improve salt tolerance in plants by increasing levels of osmolytes. For ex-

ample, osmolyte concentrations have been manipulated by constitutively expressing genes for a protective protein (Xu et al., 1996) or genes for key enzymes involved in the production of osmosolutes occasionally associated with protective activities (Tarczynski et al., 1993; Thomas et al., 1995; Hayashi et al., 1997; Karakas et al., 1997).

Photosynthesis, one of the most important metabolic pathways in plants, is a target of salt stress. Abscissic acid produced in response to salt stress (Leung et al., 1994) decreases turgor in guard cells and thus limits the CO<sub>2</sub> available for photosynthesis. During water stress, reduction of chloroplast stromal volume (Gupta and Berkowitz, 1988) and generation of active oxygen species (Price and Hendry, 1991) also are thought to play important roles in inhibiting photosynthesis.

Analysis of salt-tolerant mutants may help researchers to elucidate the mechanisms of salt tolerance and to increase resistance to salt stress in sensitive species. Several Arabidopsis mutants with altered responses to salt have been reported, including mutants tolerant to salt during germination (Saleki et al., 1993; Werner and Finkelstein, 1995) and mutants that are salt hypersensitive (Wu et al., 1996). However, no mutants have been reported whose photoautotrophic growth is salt tolerant. To address the mechanisms of salt

<sup>1</sup>To whom correspondence should be addressed. E-mail hirokazu@u-shizuoka-ken.ac.jp; fax 81-54-264-5584.

tolerance in photoautotrophic growth of plants and their application to plant improvement, we subjected *Arabidopsis* plants to mutagenesis, and we selected mutant seedlings surviving under salt stress.

## RESULTS

### Selection of Mutants Capable of Photoautotrophic Growth during Salt Stress

Instead of widely used media, such as Murashige and Skoog (MS) medium enriched with  $K^+$  (Murashige and Skoog, 1962), a low-salt mineral medium containing essential elements was used for screening mutants.  $M_2$  seedlings were germinated on low-salt mineral medium and then transferred to 200 mM NaCl in solid medium for 2 weeks. Under these conditions, cotyledons of nonmutagenized plants completely bleached and died. By using this screen we have succeeded in isolating two salt-tolerant mutant lines designated *pst* (for *photoautotrophic salt tolerance*) from a total of 148,300 plants from 22,300 lines mutagenized through either ethyl methanesulfonate treatment or T-DNA tagging (Table 1). Although two mutants designated *pst1* and *pst2* were obtained, we focused on *pst1* in this study.

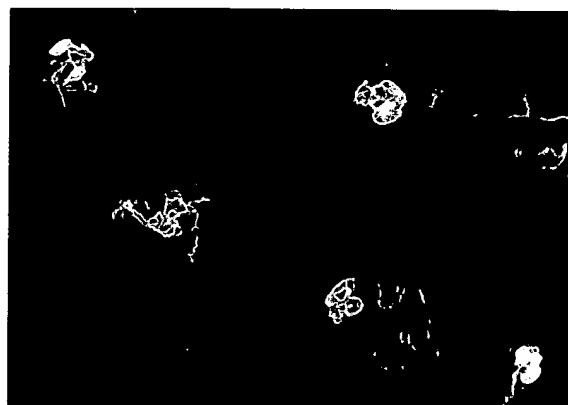
Individuals showing the phenotype of *pst1* were selected from *pst1* progeny for seven generations. However, not all progeny of *pst1* parents showed the phenotype of the *pst1* mutant. When grown photoautotrophically, these resistant plants survived for 2 weeks on medium containing 200 mM NaCl or for 1 month on medium containing 100 mM NaCl (Figures 1A and 1B). Nonmutagenized plants apparently died under the same conditions. Salt-tolerant and salt-sensitive plants bore the same percentage of salt-tolerant progeny, indicating that the plants were homozygous at the *pst1* locus. Survival rates of *pst1* plants at NaCl concentrations from 100 to 300 mM, at which all wild-type plants died, were nearly constant at 20% (Figure 2). Therefore, the penetrance of *pst1* phenotype is concluded to be incomplete at ~20%. Most *pst1* plants were unable to survive on 400 mM NaCl medium (Figure 2). The salt tolerance of *pst1* mutants was

**Table 1.** Selection of *Arabidopsis pst* Mutants

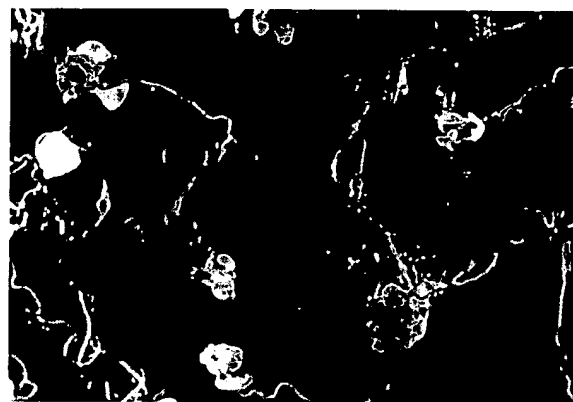
Ecotype	Mutagenesis	No. Screened Lines	No. Screened Plants	No. <i>pst</i> Lines
Columbia	Ethyl methanesulfonate	17,400	109,100	2
Wassilewskija	T-DNA tagging <sup>a</sup>	4,900	39,200	0
Total		22,300	148,300	2

<sup>a</sup> Made by Feldmann (1991) and supplied by the Nottingham *Arabidopsis* Stock Centre.

**A**



**B**



**Figure 1.** Seedlings Maintained on 100 mM NaCl Medium for 1 Month.

(A) Wild-type seedlings.

(B) *pst1* seedlings.

Seedlings were germinated and grown in the light for 7 days without salt stress and then transferred to NaCl-containing medium and maintained at a light intensity of  $39 \mu\text{mol m}^{-2} \text{sec}^{-1}$ . Under these growth conditions, wild-type plants did not survive but *pst1* mutants did. Bar in (B) = 1 cm for (A) and (B).

maintained throughout their development from seedlings to mature plants (data not shown). There were no significant morphological differences between *pst1* and wild-type lines when they were grown without stress (data not shown).

### Mapping of the *pst1* Locus

Two factors complicated genetic analysis of the *pst1* locus: (1) the phenotype of *pst1* mutation exhibited the incomplete penetrance; and (2) the mutant occurs in ecotype Columbia,

whose usual partner for crossing (ecotype Landsberg *erecta*) is less sensitive to salt stress than is ecotype Columbia. We found that salt sensitivity of ecotype RLD is similar to that of ecotype Columbia. Polymorphism of genetic markers between RLD and Columbia allowed us to map the *pst1* allele by using the simple sequence length polymorphism method (Bell and Ecker, 1994) with 82 plants. The *pst1* allele is located at the top of chromosome 3 at  $6.71 \pm 3.28$  centimorgans from *nga162* and  $9.8 \pm 3.9$  centimorgans from *AthCHIB*. No other loci related to environmental stress have been found in this area. It is concluded that *pst1* is a previously undescribed locus.

#### Sensitivity to Salt during Germination

Both *pst1* and nonmutant lines were sensitive to salt during the imbibition period of germination (Figure 3). In contrast, previously reported mutants were capable of germinating under salt stress but were sensitive to salt during vegetative growth (Saleki et al., 1993; Werner and Finkelstein, 1995). Although germination of *pst1* and wild-type seeds was inhibited by salt, the seeds remained viable, germinating when transferred to standard MS medium.

#### Salt Tolerance in *pst1* Is Not Due to Osmolyte Accumulation

Proline is thought to function as an osmoregulatory solute in stress adaptation in many plants (Delauney and Verma,

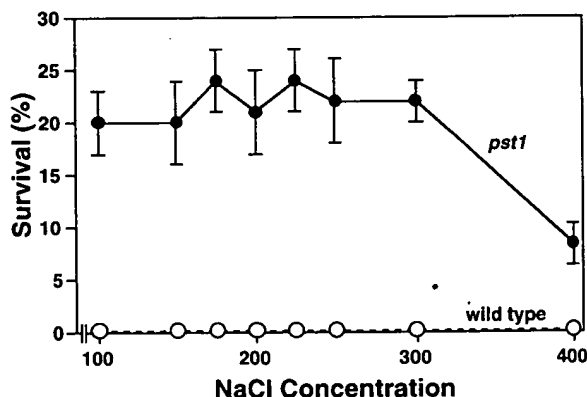


Figure 2. Survival Rates of *pst1* Plants at Increasing Concentrations of NaCl in Mineral Medium.

Shown are the survival rates of *pst1* plants (filled circles) at concentrations above which all wild-type plants (open circles) died. One-week-old seedlings were transferred to NaCl-containing medium and maintained for periods during which all wild-type plants died. The data are averages of the results obtained from three independent Petri dishes.

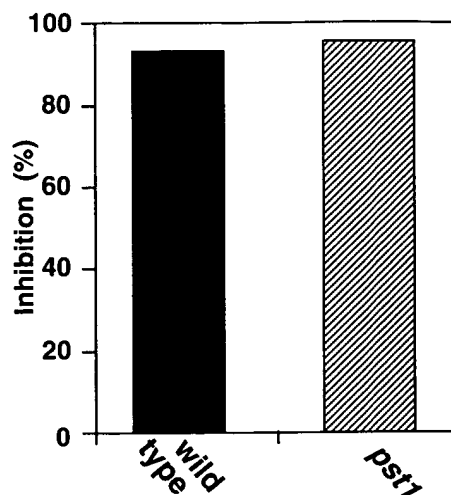


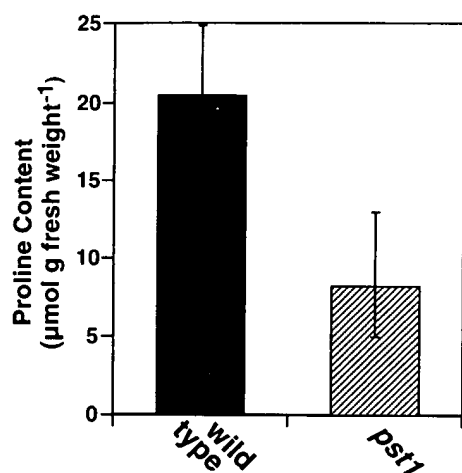
Figure 3. Inhibition Rate of Germination of Seeds on MS Medium Containing 200 mM NaCl.

Germinating seeds were counted after 10 days on the NaCl-containing medium. Seeds incapable of germinating without salt stress were excluded. The data are averages of three individual experiments.

1993), and it is known to play a role in adaptation of Arabidopsis to salt stress (Yoshida et al., 1995). These observations indicated that increased salt tolerance in *pst1* plants might be due to increased levels of cytoplasmic proline. In all plants cultured on MS medium for 3 weeks without salt stress, proline was undetectable. Subsequent exposure to 200 mM NaCl for 5 days caused proline accumulation of up to  $20 \mu\text{mol g fresh weight}^{-1}$  in wild-type plants and up to  $8 \mu\text{mol g fresh weight}^{-1}$  in *pst1* progeny (Figure 4). When *pst1* plants showing the salt-tolerant phenotype were analyzed, similar results were obtained (data not shown). Because the induced levels of proline in the *pst1* mutants are lower than those in the parental lines, proline accumulation does not confer salt tolerance to the *pst1* mutants. The reduced accumulation of proline in the *pst1* mutants may indicate that *pst1* plants have gained a different mechanism of salt tolerance.

#### Accumulation of $\text{Na}^+$ and Uptake of Other Cations

The cation content of *pst1* plants before and after exposure to salt stress was determined. The  $\text{Na}^+$  content of both lines increased 11.5 times in the presence of 200 mM NaCl (Figure 5A). The concentration of  $\text{K}^+$  in wild-type and *pst1* plants decreased under salt stress (Figure 5B). With or without salt stress, levels of  $\text{Ca}^{2+}$  (Figure 5C) and  $\text{Mg}^{2+}$  (Figure 5D) were not significantly different between nonmutagenized and *pst1* mutant lines. MS medium containing 19.8 mM  $\text{K}^+$  (Murashige and Skoog, 1962) slightly reduced salt stress compared with



**Figure 4.** Accumulation of Proline in Leaves of Plants Growing in MS Medium Containing 200 mM NaCl.

Plants were cultured without salt stress for 3 weeks before exposure to NaCl. Contents of proline as a major osmoregulatory solute were determined after a 5-day incubation on medium containing 200 mM NaCl. The data presented are averages of the results obtained from three individual experiments.

mineral medium containing 7.5 mM K<sup>+</sup> (data not shown). Under all conditions tested, there were no significant differences in K<sup>+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> contents and the ratio of K<sup>+</sup> to Na<sup>+</sup> (Figure 5E) between wild-type and *pst1* plants. Therefore, the mechanism of salt tolerance in *pst1* cannot be explained by alteration of cation uptake ability.

#### Salt Stress Is Increased by Moderate Light Intensities

We found that light intensity affected the sensitivity of wild-type seedlings to salt. Seedlings grown without stress were exposed to NaCl under different intensities of light. In the absence of salt stress, light at moderate intensities (26 and 39 μmol m<sup>-2</sup> sec<sup>-1</sup>) did not inhibit seedling growth. In contrast, moderate light caused severe damage to wild-type plants grown under salt stress (Figure 6). Seedlings grown for 7 days with 200 mM NaCl and 26 or 39 μmol m<sup>-2</sup> sec<sup>-1</sup> light did not survive, whereas ~90% of seedlings grown in 13 μmol m<sup>-2</sup> sec<sup>-1</sup> light survived. Nearly 100% of salt-stressed seedlings grown in low light (6.5 μmol m<sup>-2</sup> sec<sup>-1</sup>) or in the dark survived. The survival rates of wild-type and *pst1* plants were different at 26 μmol m<sup>-2</sup> sec<sup>-1</sup> or higher light intensities; the mutant plants were more tolerant than were the wild-type plants (Figure 6). In the presence of 200 mM NaCl, both wild-type and *pst1* plants cultured in the dark without sugar remained viable and green for at least 3

months but did not grow. In contrast, the plants bleached and died under the same conditions when the salt stress was eliminated (data not shown).

The expression of members of the gene families of *RBCS*, which codes for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and *CAB* (also known as *LHCB*), which codes for chlorophyll *a/b* binding protein, without salt stress increased and kept plateaus with elevating light intensities (Figures 7A and 7B). In contrast, *RBCS* or *CAB* expression in wild-type plants showed maximal at ~10 μmol m<sup>-2</sup> sec<sup>-1</sup> in the presence of 200 mM NaCl but diminished with increasing intensities of light (Figures 7A and 7B). Therefore, salt stress was strengthened even by moderate intensities of light. This indicates that salt stress may be mediated by photosynthetic activity. However, salt stress interfered less with the expression of photosynthesis genes in the mutant plants (Figures 7A and 7B).

#### Tolerance to Superoxide Radicals

The apparent coupling of salt sensitivity with photosynthesis led us to speculate that salt may promote the generation of active oxygen species through photosynthesis. The herbicide methyl viologen, commonly known as paraquat, generates superoxide radicals during photosynthesis that cause damage to photosystems I and II (Dodge, 1994). The *pst1* line was more tolerant of methyl viologen than was the wild type (Figures 8A and 8B). Plants that were sensitive to methyl viologen began to bleach after 2 days of exposure to the herbicide. The survival rate of *pst1* plants treated with the herbicide for 7 days was 10 times higher than that of non-mutagenized plants (Figure 8C). The damage to plants caused by the herbicide also was evaluated by measuring electrolyte leakage. Reduced electrolyte leakage from *pst1* plants exposed to methyl viologen (Figure 9) indicated that the *pst1* line was more tolerant of the herbicide.

#### Activity of Active Oxygen-Scavenging Enzymes

It is an intriguing question whether active oxygen species are detoxified rapidly in *pst1* mutants or whether the metabolic machinery in the mutants in some way tolerates active oxygen. We determined the activities of superoxide dismutase (SOD), which catalyzes the conversion of superoxide to hydrogen peroxide, and of ascorbate peroxidase (APX), which is involved in removal of hydrogen peroxide. When plants were exposed to 200 mM NaCl for 5 days, the activity of both enzymes was elevated in the *pst1* line compared with wild types (Figures 10A and 10B). These results indicate that salt stress enhances the production of active oxygen species and that active oxygen species detoxified more efficiently in the *pst1* line, resulting in enhanced stress tolerance.

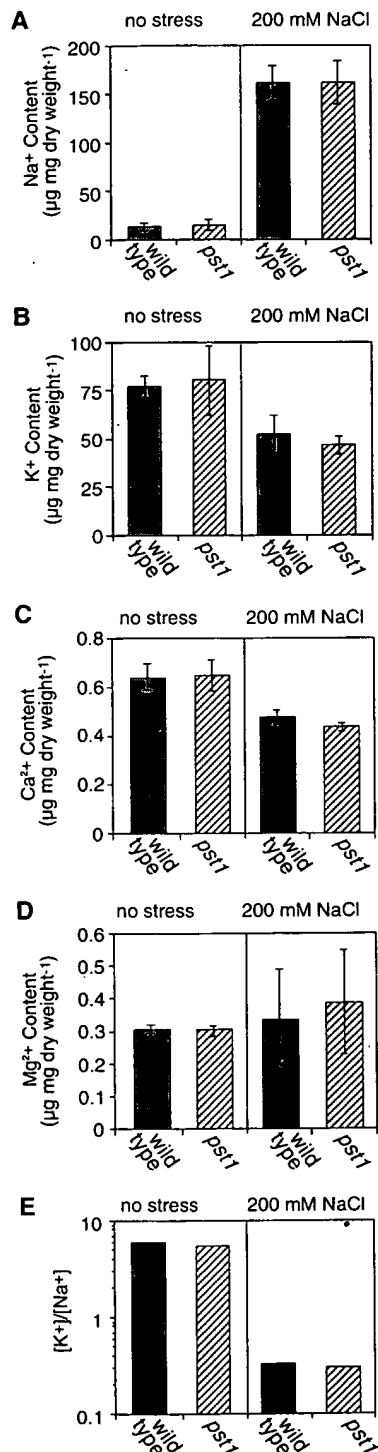


Figure 5. Concentrations of Cations in Leaves of Plants on MS Medium Containing 200 mM NaCl.

### Tolerance of *pst1* Mutants to High Light Intensity

The association of increased SOD and APX activity with salt tolerance in *pst1* led us to speculate that these mutants also would show increased tolerance to high-intensity light, which promotes active oxygen production. It is known that high-intensity light affects the chlorophyll fluorescence emission from leaves. A simple fluorescence parameter  $F_v/F_m$  ( $F_v = F_m - F_o$ ;  $F_m$ , maximal fluorescence;  $F_o$ , steady state fluorescence) can be used routinely to estimate changes in the quantum yield of noncyclic electron transport (Genty et al., 1989). As shown by  $F_v/F_m$  measurement, photosystem II suffered less damage in *pst1* mutants than in wild-type plants when exposed to high-intensity light ( $675 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ) for 3 hr (Figure 11).

### Tolerance of *pst1* Mutants to LiCl or CsCl

It is known that  $\text{Cs}^+$  enters through  $\text{K}^+$  influx channels in higher plants (Very et al., 1995; Ichida et al., 1997) and that  $\text{Na}^+$ -hypersensitive mutants of yeast are highly sensitive to  $\text{Li}^+$  (Mendoza et al., 1994). Plants grown without salt stress for 7 days were transferred to medium containing either 20 mM LiCl or 10 mM CsCl. The wild-type plants were less viable on the 10 mM CsCl medium than were the *pst1* plants (Figures 12A and 12B). Wild-type plants exposed to LiCl or CsCl for 12 days died, whereas ~40% of *pst1* plants grown under the same conditions survived (Figures 12C and 12D). After a 5-day exposure to  $\text{Li}^+$  or  $\text{Cs}^+$ , the levels of  $\text{Li}^+$  or  $\text{Cs}^+$  were similar in the wild-type and *pst1* lines (Figure 13). These results indicate that the capacity for cation uptake in *pst1* mutants is not different from that of wild-type plants.

### DISCUSSION

The near constant percentages of surviving *pst1* plants between NaCl concentrations of 100 and 300 mM (Figure 2) indicate the incomplete penetrance of the *pst1* phenotype (Brown, 1992). The profile of survival rates as a function of

Plants were cultured without salt stress for 3 weeks before exposure to NaCl, after which they were maintained for 5 days on media without (left) or with (right) 200 mM NaCl. The data presented are means of three individual experiments.

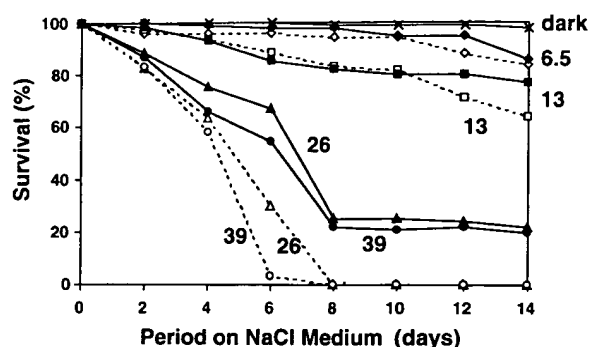
(A)  $\text{Na}^+$  leaf content.

(B)  $\text{K}^+$  leaf content.

(C)  $\text{Ca}^{2+}$  leaf content.

(D)  $\text{Mg}^{2+}$  leaf content.

(E) Ratios of  $\text{K}^+$  to  $\text{Na}^+$ .



**Figure 6.** Influence of Moderate Light Intensities on Survival of Plants Cultured in the Presence of 200 mM NaCl.

Seven-day-old seedlings were transferred to mineral medium containing 200 mM NaCl and incubated under different intensities of continuous white fluorescent light. The values next to the lines indicate light intensities in micromoles per square meter per second. The open symbols and dotted lines are for wild-type plants, and the closed symbols and solid lines are for *pst1* plants.

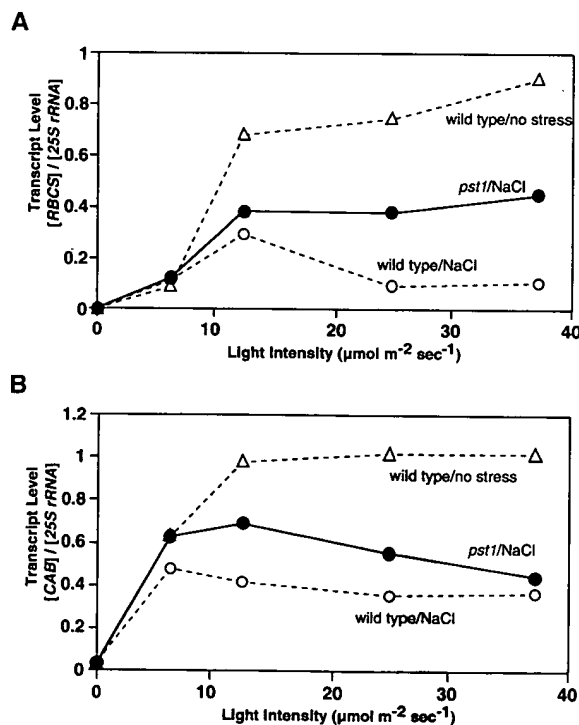
salt concentrations (Figure 2) shows that the upper threshold is near 300 mM, above which osmotic pressure or  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations are high enough to kill the mutant plants. The incomplete penetrance could result from insufficient activity of enzymes that detoxify active oxygen species for conferring complete salt tolerance on all *pst1* plants. Because activities of SOD or APX in individual *pst1* plants in each generation that showed the salt-tolerant and wild-type phenotypes were nearly identical within standard deviations of  $\sim 10\%$  (data not shown), we speculate that events that caused death were complex, possibly including interactions with other factors.

When plants were exposed to salt stress, they accumulated the osmolyte proline (Figure 4). Rubisco, located in the chloroplast stroma, is the most abundant protein on earth and constitutes up to 50% of total chloroplast protein (Ellis, 1979). It has been reported that the massive accumulation of amino acids or their derivatives under conditions of water depletion may be associated with increased protein breakdown (Nover et al., 1989; Callis, 1995). The expression of the photosynthesis genes *RBCS* and *CAB* was strongly inhibited by salt stress (Figures 7A and 7B). We speculate that the reduction of *RBCS* expression during salt stress may be necessary for terminating incorporation of amino acids into proteins. Decreased protein synthesis and enhanced protein degradation could yield the high concentrations of free amino acids needed to maintain osmotic pressure in salt-stressed plants.

Proton-coupled  $\text{K}^+$  transport generates osmotic pressure in cells and in their intracellular compartments (Läuchi and Bielecki, 1983). Increases in osmotic pressure may contribute to salt tolerance. The *sos1* (for salt-overly sensitive) mu-

tants are defective in high-affinity  $\text{K}^+$  uptake and in low-affinity  $\text{Na}^+$  uptake (Ding and Zhu, 1997). The *pst1* mutants accumulated  $\text{Cs}^+$ , which is known to enter through  $\text{K}^+$  influx channels (Very et al., 1995; Ichida et al., 1997), to the same level as wild-type plants (Figure 13B). This observation confirms that the activity of  $\text{K}^+$  influx channels is not enhanced in the *pst1* mutants.

It has been shown that  $\text{Ca}^{2+}$  is required to maintain or enhance the selective import of  $\text{K}^+$  into plants exposed to high concentrations of external  $\text{Na}^+$  (Lahaye and Epstein, 1969). Interacellular  $\text{Ca}^{2+}$  signaling through a calcineurin-like pathway has been reported to play a crucial role in salt tolerance (Liu and Zhu, 1998). Furthermore, the  $\text{Ca}^{2+}$ -dependent protein kinases CDPK1 and CDPK1a are involved in stress signal transduction (Sheen, 1996). Salt tolerance of *pst1* does not appear to be related to changes in total intracellular concentrations of  $\text{Ca}^{2+}$ , because  $\text{Ca}^{2+}$  content in *pst1* and wild-type plants was equivalent under salt stress (Figure 5C).

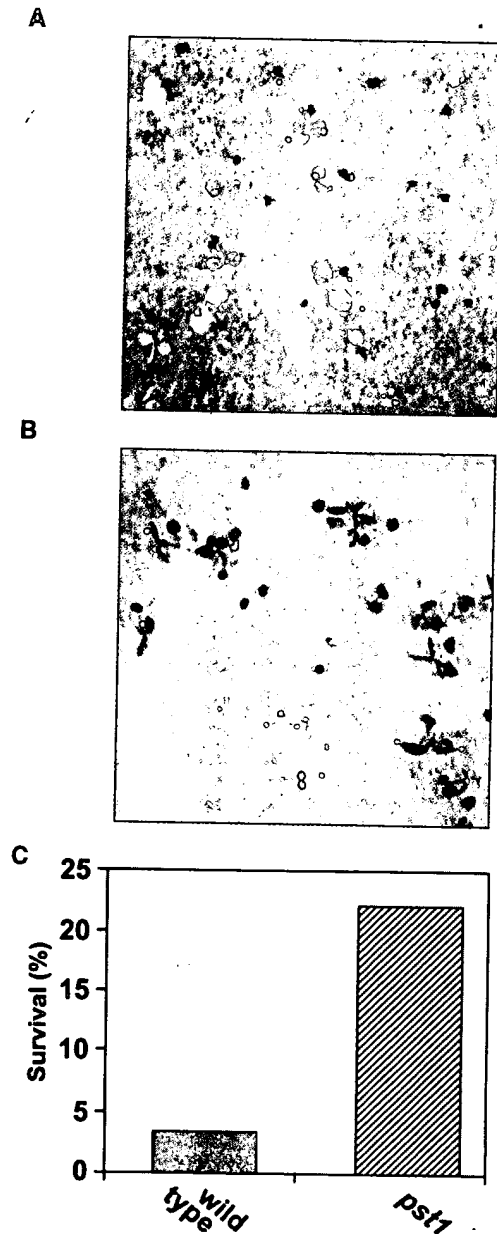


**Figure 7.** Effect of Moderate Light Intensities on Expression of *RBCS* and *CAB* in Leaves of Plants Grown on MS Medium Containing 200 mM NaCl.

Three-week-old plants were transferred to the high-salt medium and incubated for 5 days under different intensities of continuous white fluorescent light. Transcript levels were normalized by using the 25S rRNA.

(A) *RBCS* transcripts.

(B) *CAB* transcripts.



**Figure 8.** Sensitivity of Plants to Superoxide Radicals Generated with Methyl Viologen.

Superoxide radicals were generated by treatment with 30  $\mu\text{M}$  methyl viologen for 7 days under moderate light intensity ( $29 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ). The seedlings were treated on filter paper soaked in methyl viologen dissolved in distilled water. Seedling survival rates are means of three individual experiments.

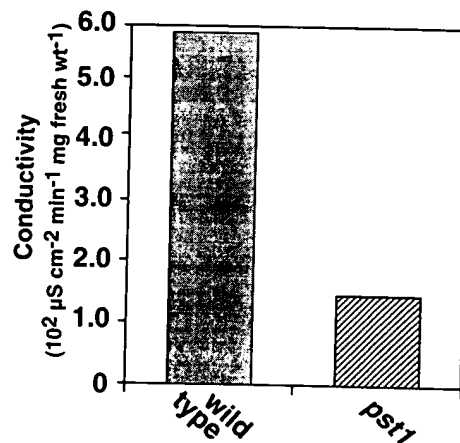
(A) Wild type.

(B) *pst1*.

(C) Survival rate.

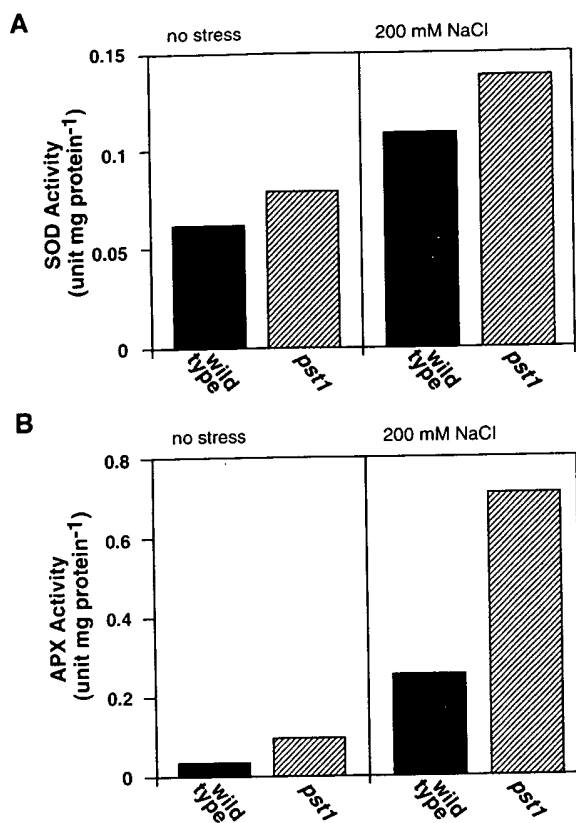
We found that light, even at moderate intensities, increased salt stress (Figure 6). We also have observed that salt-stressed plants cultured without sugar in the dark remain alive and green for at least 3 months but do not grow. In contrast, plants bleach and die under the same conditions when salt stress is eliminated (data not shown). Changes in light intensity have dramatic effects on the salt sensitivity. It is possible that salt stress in the dark causes seedling dormancy. This response could result from a signal transduction pathway activated by salt exposure in the dark, possibly involving abscisic acid, which induces dormancy in plants. The survival rates (Figure 6) and levels of transcripts for photosynthesis genes (Figures 7A and 7B) were significantly higher in *pst1* plants than they were in wild-type plants under salt stress and increasing intensities of light, leading to the speculation that *pst1* plants are more tolerant to active oxygen species, which are generated under higher intensities of light.

*pst1* seedlings were more tolerant than wild-type plants to methyl viologen and high light intensity as well as  $\text{Li}^+$  and  $\text{Cs}^+$ . Active oxygen species reportedly have been generated by drought (Eltner, 1991), herbicide treatment (Asada, 1994), and cold stress (Malan et al., 1990). Increases in both SOD and APX activities in *pst1* mutants support the suggestion that the *pst1* mutation increases active oxygen-scavenging activity and thus confers tolerance to general environmental stresses. The Cu/Zn-SOD in chloroplasts is the major SOD detected in plant leaves (Asada et al., 1973). Ascorbate peroxidase, which catalyzes the conversion of



**Figure 9.** Electrolyte Leakage from Seedlings Treated with Methyl Viologen.

Plants were soaked in 10 nM methyl viologen solution in 24-well plates (Stem, Tokyo, Japan) under moderate light intensity ( $29 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ). Leakage values were calculated from slopes maintained over 16 hr. The data presented are means of three individual experiments. S, sec; wt, weight.



**Figure 10.** Activities of SOD and APX in Leaves of Plants Maintained on 200 mM NaCl MS Medium.

Plants were grown for 3 weeks without salt stress before exposure to NaCl for 3 days. The data are averages of three individual experiments. (A) SOD. (B) APX.

H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O, also is found in chloroplasts (Miyake and Asada, 1992). With or without salt stress, the activities of SOD and APX in *pst1* were ~1.3 and 3 times the activities of the wild type, respectively (Figures 10A and 10B). A 30 to 100% elevation of SOD activity in transgenic tobacco and alfalfa has been reported to confer tolerance to oxidative stress (Gupta et al., 1993; Van Camp et al., 1994) and freezing stress (McKersie et al., 1993). Therefore, the detected increase of SOD and APX activities in *pst1* plants may play an important role in the enhanced tolerance of the mutants to salt and other stresses.

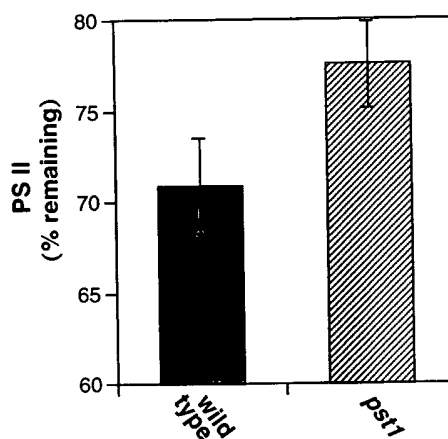
The recessive nature of the *pst1* mutation indicates that the potential for salt tolerance is blocked in wild-type *Arabidopsis*. Therefore, land plants may have suppressed but not lost the capacity for salt tolerance during their evolution from ancestral organisms exposed to salt stress or more severe oxidative stress. Although plants in the *pst1* line have a

single mutation, activities of at least two enzymes for detoxifying active oxygen species were enhanced in this mutant. Thus, the *PST1* gene may regulate the expression of a series of genes involved in active oxygen detoxification.

## METHODS

### Plant Growth

Seeds (*Arabidopsis thaliana* ecotype Columbia) were surface-sterilized in 70% ethanol for 1 min and then in 5% NaCl containing 0.05% Tween 20 for 10 min, followed by five washes with sterile distilled water. The seeds were sown in plastic Petri dishes containing solidified gellan gum (0.2% [w/v]; Kelco, San Diego, CA) with either low-salt mineral medium (5 mM KNO<sub>3</sub>, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 50 μM Fe-EDTA, 70 μM H<sub>3</sub>BO<sub>4</sub>, 14 μM MnCl<sub>2</sub>, 0.5 μM CuSO<sub>4</sub>, 1 μM ZnSO<sub>4</sub>, 0.2 μM Na<sub>2</sub>MoO<sub>4</sub>, 10 μM NaCl, and 0.01 μM CoCl<sub>2</sub>) for screening of mutants or Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) for synchronized growth of seedlings. Unless otherwise noted, plants were grown axenically at 22°C for 7 days under moderate intensity (26 μmol m<sup>-2</sup> sec<sup>-1</sup>) of continuous white fluorescent light. Seedlings were then transferred to new medium of the same composition, treated with NaCl or other stresses, and kept under the same growth conditions for the indicated periods.



**Figure 11.** Effect of High Light Intensity on Photosystem II in Leaves.

Plants were cultured for 4 weeks without salt stress. Experiments were conducted under high light intensity (675 μmol m<sup>-2</sup> sec<sup>-1</sup>) at 20°C for 3 hr. Remaining photosystem II (PS II) activities were estimated by measuring chlorophyll fluorescence immediately after exposure to irradiance. Leaves exhibited  $F_v/F_m$  values between 0.75 and 0.85, which are similar to values reported for photosystem II in unstressed plants (Genty et al., 1989). The ordinate indicates  $F_v/F_m$  values normalized to those of untreated leaves (average  $F_v/F_m$  = 0.8). The data are averages of three individual experiments.



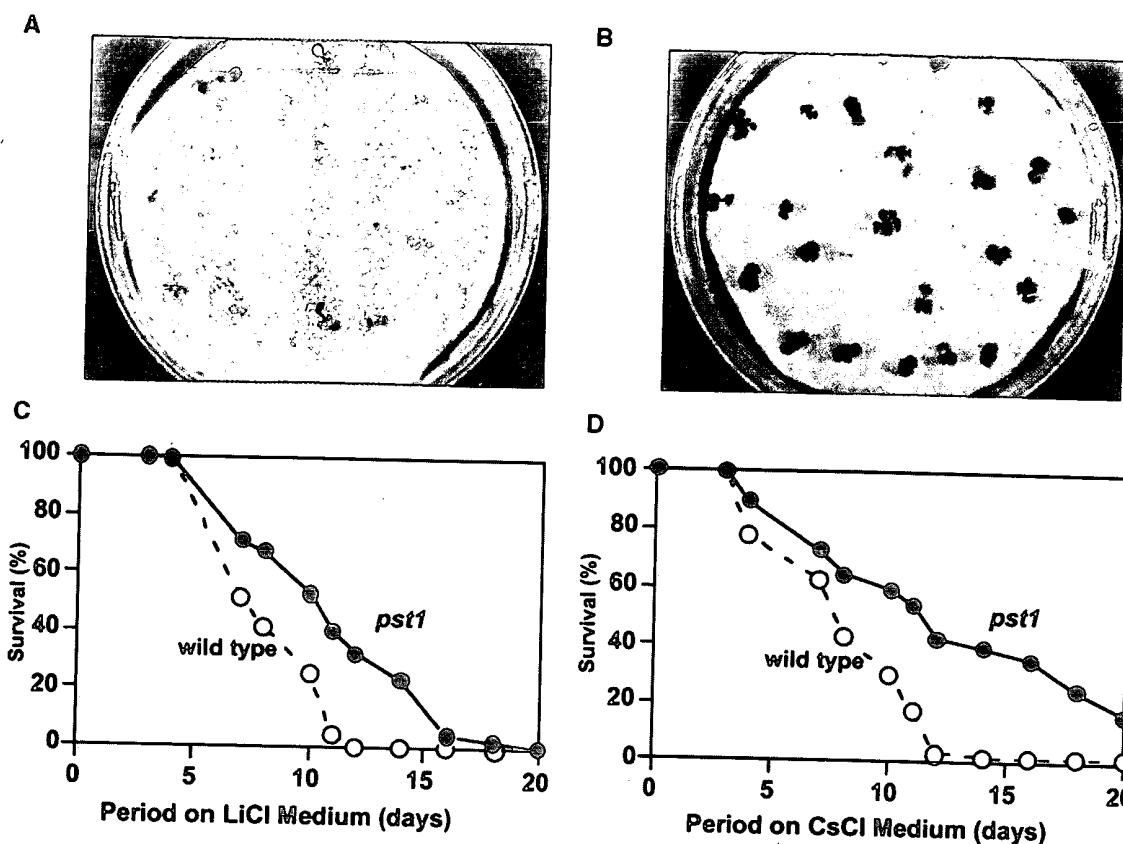


Figure 12. Influence of LiCl and CsCl on Survival of Seedlings.

(A) and (B) Sensitivity of wild-type (A) and *pst1* (B) plants grown on MS medium containing 10 mM CsCl for 7 days under light at  $19.5 \mu\text{mol m}^{-2} \text{sec}^{-1}$  is shown.

(C) and (D) Seven-day-old seedlings were transferred to MS medium containing 20 mM LiCl (C) or 10 mM CsCl (D). Seedlings were incubated at a light intensity of  $39 \mu\text{mol m}^{-2} \text{sec}^{-1}$ . The survival rates presented are means of three individual experiments.

#### Genetic Analysis

To map the photoautotrophic salt tolerance *pst1* locus, the *pst1* mutant line was crossed with ecotype RLD, and the  $F_2$  progeny were screened for salt tolerance. Using standard methods (Bell and Ecker, 1994), we found the *pst1* locus to be linked to the simple sequence length polymorphism marker *nga6* on chromosome 3. Primers for this analysis were purchased from Research Genetics (Huntsville, AL). Map distances were calculated as described by Koornneef and Stam (1992).

#### Determination of Proline and Protein Concentrations

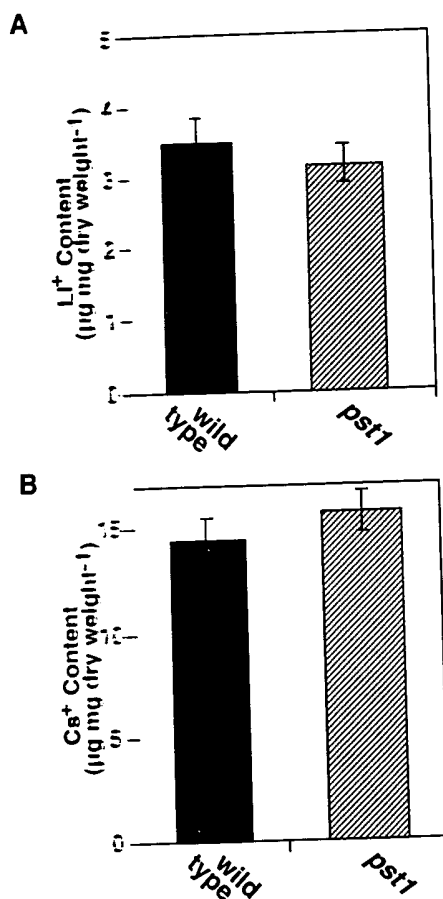
Proline was extracted from leaves, and its concentration was determined by the method reported by Sanada et al. (1995). Protein concentrations were determined using a BCA (bicinchoninic acid) protein assay kit (Pierce Chemical Co., Rockford, IL), according to the manufacturer's instructions.

#### Cation Contents

Cation contents were determined by atomic absorption spectrometry. Harvested leaves were washed with distilled water and dried at  $60^\circ\text{C}$  for 7 days in 1.5-mL tubes. Samples then were weighed and ashed at  $500^\circ\text{C}$  overnight in a muffle furnace (Electric Muffle Furnace KL-160; Advantec Toyo, Tokyo, Japan). The ash was solubilized by heating in 1 M HCl. Cation contents were determined by using a polarized Zeeman atomic absorption spectrophotometer Z-8000 (Hitachi, Ltd., Hitachi, Japan).

#### Determination of Transcript Levels

RNA was isolated from leaves by using Isogen (Wako, Tokyo, Japan) according to the manufacturer's instructions. RNA gel blot analysis was performed following Sambrook et al. (1989). Probes used for hybridization were a 393-bp *NcoI*-*NsiI* fragment of *RBSC-3B* (Krebbbers et al., 1988) from expressed sequence tag clone 36G877, supplied by the Arabidopsis Biological Resource Center (Columbus, OH); a



**Figure 13.** Concentrations of Li<sup>+</sup> and Cs<sup>+</sup> in Plant Leaves.

(A) Plants grown in 10 mM LiCl for 5 days under light at 39 µmol m<sup>-2</sup> sec<sup>-1</sup>.

(B) Plants grown in 10 mM CsCl for 5 days under light at 39 µmol m<sup>-2</sup> sec<sup>-1</sup>.

Plants were grown for 3 weeks before transfer to MS medium containing LiCl or CsCl. The data presented are means of three individual experiments.

592-bp HindIII-SacI fragment of *CAB1* (Leutwiler et al., 1986) of DNA amplified by polymerase chain reaction with genomic DNA of Arabidopsis ecotype Columbia; and a 3.7-kb fragment for 25S rRNA from the clone VER18 containing DNA from *Vicia faba* (Yakura and Tanifuji, 1983).

#### Treatment with Methyl Viologen and Measurement of Electrolyte Leakage

Seedlings grown on filter paper wetted with distilled water were soaked in 30 µM methyl viologen and incubated until plants became

bleached. Seedlings also were exposed to 10 nM methyl viologen for measuring conductivity to evaluate electrolyte leakage. The conductivity of the ambient solution was determined using a twin conductivity meter B-173 (Horiba, Kyoto, Japan).

#### Determination of Superoxide Dismutase and Ascorbate Peroxidase Activities

Plant leaves were homogenized in 0.1 M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, at 4°C and centrifuged at 4000g for 15 min at 4°C. The supernatant was filtered through filter paper (No. 1; Whatman) and subjected to gel filtration in Cellulofine GH-25-m (Chisso, Osaka, Japan) preequilibrated with 50 mM Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub>, pH 10.2 (Spychalla and Desborough, 1990). Superoxide dismutase (SOD) activity was determined using an SOD-525 assay kit (OXIS International, Inc., Portland, OR) according to the manufacturer's instructions. The assay is based on the catalytic conversion of SOD to autoxidize 5.6.6a,11b-tetrahydroxybenzo(C) fluorene in alkaline solution and is monitored by measurement of absorbance of the resultant chromophore at 525 nm.

Ascorbate peroxidase (APX) was assayed as described previously by Chen and Asada (1990). The reaction mixture contained 50 mM K-phosphate, pH 7.0, 0.5 mM hydrogen peroxide, and 1 mM ascorbate. One and one-half to 11.5 sec after initiating the reaction by addition of hydrogen peroxide, we measured the activity of APX by the decrease in absorbance at 290 nm (absorbance coefficient for ascorbate of 2.8 mM<sup>-1</sup> cm<sup>-1</sup>).

#### Chlorophyll Fluorescence

Chlorophyll fluorescence from leaves was determined by using monitoring system MFMS II (Hansatech, King's Lynn, UK). Detached leaves were placed on solid mineral medium in the assay chamber and exposed to high intensity light (675 µmol m<sup>-2</sup> sec<sup>-1</sup>) at 20°C for 3 hr before measurement. Leaves did not become dry over this period.

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